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Ralstonia solanacearum Iron Scavenging by the Siderophore Staphyloferrin B Is Controlled by PhcA, the Global Virulence Regulator

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PhcA is a transcriptional regulator that activates expression of multiple virulence genes in the plant pathogen *Ralstonia solanacearum*. Relative to their wild-type parents, *phcA* mutants overproduced iron-scavenging activity detected with chrome azurol S siderophore detection medium. Transposon mutagenesis of strain AW1-PC (*phcA1*) generated strain GB6, which was siderophore negative but retained weak iron-scavenging activity. The *ssd* gene inactivated in GB6 encodes a protein similar to group IV amino acid decarboxylases, and its transcription was repressed by iron(III) and PhcA. *ssd* is the terminal gene in a putative operon that also appears to encode three siderophore synthetase subunits, a integral membrane exporter, and three genes with no obvious role in siderophore production. A homologous operon was found in the genomes of *Ralstonia metallidurans* and *Staphylococcus aureus*, both of which produce the polycarboxylate siderophore staphyloferrin B. Comparison of the siderophores present in culture supernatants of *R. solanacearum*, *R. metallidurans*, and *Bacillus megaterium* using chemical tests, a siderophore utilization bioassay, thin-layer chromatography, and mass spectroscopy indicated that *R. solanacearum* produces staphyloferrin B rather than schizokinen as was reported previously. Inactivation of *ssd* in a wild-type AW1 background resulted in a mutant almost incapable of scavenging iron but normally virulent on tomato plants. AW1 did not produce siderophore activity when cultured in tomato xylem sap, suggesting that the main location in tomato for *R. solanacearum* during pathogenesis is iron replete.

Although virtually all microorganisms require iron, this common element usually is present in forms that are biologically unavailable to aerobic organisms (1, 15). Bacteria often respond to iron deprivation by producing one or more low-molecular-mass siderophores and outer-membrane siderophore receptor proteins to scavenge iron(III) from their surroundings (1, 47). Based on the ligands that participate in chelating iron, the better-known siderophores often are classified as either catecholates or hydroxamates (1). For example, some *Escherichia coli* strains make enterobactin, a cyclic catecholate, and aerobactin, a linear citrate-containing dihydroxamate (15, 40). Schizokinen, which was originally described as the siderophore produced by *Bacillus megaterium* ATCC 19231 (6), has a structure very similar to aerobactin. A third group of especially hydrophilic, citrate-containing siderophores with α-hydroxycarboxylate ligands has been recognized, and they are referred to as either polycarboxylates or complexones. Polycarboxylate siderophores include rhizoferrin, staphyloferrin B, vibrioferrin, and achromobactin. Some bacteria can also use citrate to scavenge iron, although it is a weak chelator compared to dedicated siderophores (1, 15).

Ralstonia solanacearum is a soilborne pathogen that causes lethal wilt diseases of many plants around the world. After entering plant roots, the pathogen invades the water-conducting xylem vessels and rapidly spreads throughout the vascular system. Systemic colonization requires that the pathogen secrete an array of virulence factors, including the high-molecular-mass extracellular polysaccharide slime (EPS1) and secreted enzymes that can degrade plant cell walls (50). Production of these factors is controlled by a complex, environmentally responsive regulatory network that uses the PhcA transcriptional regulator as part of a confinement-sensing system to directly and indirectly control multiple genes (50). Expression of multiple genes that contribute to virulence increases when bacterial density increases (e.g., within plant tissues), whereas factors that may promote survival outside of a host are more strongly expressed at low bacterial cell density.

Immediately downstream of *phcA* is the apparent ortholog of *fur* (for ferric uptake regulation), a nearly ubiquitous gene that encodes a transcriptional repressor that reduces production of siderophores and their receptor proteins under iron-replete conditions (1). We were intrigued by the juxtaposition of these two global-acting transcriptional regulators and wondered whether iron scavenging is required for virulence of *R. solanacearum*, like it is for several other plant pathogenic bacteria (16). Budzikiewicz et al. (5) reported that, like *B. megaterium*, the *R. solanacearum* type strain (ATCC 11696; also known as K60) produces the dihydroxamate citrate-containing siderophore schizokinen. Nothing was known about the genetics of schizokinen production in either bacterium, so we used transposon mutagenesis to create *R. solanacearum* mutants defective in iron-scavenging ability. Characterization of the wild type and one mutant indicated that siderophore production is negatively regulated by PhcA and is not essential for virulence on tomato. More significantly, we determined that

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR phoA*	Invitrogen
R. solanacearum		
AW1	Wild type, Nx^r, from tomato (Alabama), race 1, biovar 1, RFLP II	13
K60 (ATCC 11696)	Wild type, from tomato (North Carolina), race 1, biovar 1, RFLP II	4
GMI1000	Wild type, from tomato (French Guyana), race 1, biovar 3, RFLP I	48
SGT	Wild type, from tomato (Georgia), race 1, biovar 1	4
UW82	Wild type, from potato (Columbia), race 3, biovar 1	13
UW20	Wild type, from banana (Venezuela), race 2, biovar 1, RFLP II	4
UW30	Wild type, from tomato (Trinidad), race 1, biovar 1, RFLP II	4
UW127	Wild type, from plantain (Peru), race 2, biovar 1, RFLP II	4
UW130	Wild type, from tomato (Peru), race 1, biovar 3, RFLP I	4
UW143	Wild type, from tomato (Australia), race 1, biovar 3, RFLP I	4
AW1-PC	AW1 *phcA1*, Nx^r	4
AW1-PCΩ	AW1 *phcA*::Ω, Nx^r Sp^r	7
AW1-AI3	AW1 *solR*::Tn*5*, Nx^r Km^r	17
AW1-AI8	AW1 *soll*::Ω, Nx^r Sp^r	17
GMI1000-PCΩ	GMI1000 *phcA*::Ω, Sp^r	This study
GB40	AW1-PC *cysI*::Tn*5*-B20, Nx^r Km^r	This study
GB6	AW1-PC *ssd*::Tn*5*-B20, Nx^r Km^r	This study
AW1-GB6	AW1 *ssd*::Tn*5*-B20, Nx^r Km^r	This study
Other bacteria		
R. metallidurans CH34	Produces staphyloferrin B (previously known as *Alcaligenes eutrophus* and *R. eutropha*)	19
B. megaterium ATCC 19213	Produces schizokinen	6
Plasmids		
pBlueScript II KS+	*E. coli* cloning vector, Ap^r	Stratagene
pDrive	*E. coli* cloning vector, Km^r	Qiagen
pEX18Tc	Cloning vector for gene replacement, SacB$^+$ Tc^r	22
pLAFR3	Broad host cosmid cloning vector, Tc^r	55
pSup102-Gm::Tn*5*	Suicide plasmid, donates Tn*5*-B20, Km^r Gm^r Cm^r	54
pGMphcAΩ	pDrive::*phcA*::Ω, Km^r Sp^r	This study
pEXphcAΩ	pEX18Tc::*phcA*::Ω, Tc^r Sp^r	This study
pKeGB6	pBluescript II KS+ with DNA flanking the Tn*5*-B20 insertion in GB6, Ap^r Km^r	This study
pCGB6	pLAFR3 containing *R. solanacearum* genomic DNA that complements GB6, Tc^r	This study

[a] RFLP, Restriction fragment length polymorphism division according to Cook et al. (9). Ω is an omega interposon that encodes Sp^r, *cysI* encodes a sulfite reductase, and *ssd* encodes a siderophore synthesis decarboxylase.

the representative *R. solanacearum* strains tested make staphyloferrin B rather than schizokinen.

MATERIALS AND METHODS

Strains, culture conditions, and assays. Bacterial strains and plasmids used in this study are described in Table 1. *R. solanacearum* strains, *Ralstonia metallidurans* CH34, and *B. megaterium* ATCC 19213 (a gift from B. Rowe Byers, University of Mississippi) were cultured on BG ± 1.6% agar at 30°C (30). *E. coli* strains were grown at 37°C on Luria broth (LB) ± 1.6% agar (36). The antibiotics used were ampicillin (100 μg/ml), kanamycin (50 μg/ml), nalidixic acid (20 μg/ml), spectinomycin (50 μg/ml), and tetracycline (15 μg/ml).

The standard minimal medium was MM ± 1.6% agar (8); it was prepared by normal laboratory procedures and is low in iron. MMΔ, a variation of MM that is citrate free and very low in iron, contained the following: 10 mM K_2HPO_4, 5.5 mM KH_2PO_4, 19 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2 \cdot 2H_2O$, trace elements made without addition of iron (1,000× concentrate has 50 mM H_3BO_3, 10 mM $MnCl_2 \cdot 4H_2O$, and 1 mM each $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $Na_2MoO_4 \cdot 2H_2O$, and $ZnCl_2$ in 10 mM HCl), and 0.4% glucose. MMΔ was prepared with Milli-Q water in new plasticware and was filter sterilized. To induce siderophore production, *R. metallidurans* was cultured in succinate medium (38) and *B. megaterium* was cultured in 4× MMΔ (containing fourfold more of all ingredients except glucose). When desired, $FeCl_3 \cdot 6H_2O$ (in 10 mM HCl) was added up to a final concentration of 20 μM.

The β-galactosidase activity produced by *lacZ* transcriptional fusions in *R. solanacearum* was determined by standard methods with *o*-nitrophenyl-β-D-thiogalactopyranoside (ONPG) as the substrate (7, 36). Virulence of *R. solanacearum* on young tomato plants was assessed by using a soil-drench procedure to inoculate unwounded roots (30).

Siderophore production, detection, and characterization. The iron-scavenging activity of bacterial colonies was detected with chrome azurol S (CAS) agar (52), which was modified to support better multiplication of *R. solanacearum*. To prepare 1 liter of *Rs*CAS agar, 3.0 g of PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] and 0.6 g of NaOH were dissolved in 750 ml of H_2O, the pH was adjusted to 6.8 with 1 M NaOH, and 7.5 g of agar (Difco Bacto) was added prior to autoclaving. After cooling this mixture to 50°C, the following sterile solutions were added: 100 ml of KH_2PO_4 (0.3%) plus NH_4Cl (1%), 30 ml of non-deferrated Casamino Acids (10%; Sigma, St. Louis), 10 ml of non-deferrated proteose peptone 3 (10%; Difco), 10 ml of glucose (20%), 1 ml each of $MgSO_4 \cdot 7H_2O$ (1.0 M) and $CaCl_2 \cdot 2H_2O$ (0.1 M), and lastly 100 ml of standard CAS dye solution. Siderophore activity in culture supernatants (from minimal media or tomato sap) was detected by mixing samples 1:1 with liquid CAS reagent or CASS reagent (CAS reagent containing 4 mM 5-sulfosalicylic acid) and measuring the decrease in A_{630} 30 to 60 min later (52). A modified Csàky test (24) was used to detect hydroxamate siderophores, and 90 μM hydroxylamine hydrochloride or ≥30 μM desferrioxamine mesylate (a trihydroxamate siderophore; Sigma) was used as a positive control. The Arnow test (44) detected catechol siderophores, and a solution of 50 μM catechol was used as a positive control.

To purify *R. solanacearum* and *R. metallidurans* siderophores, culture supernatants were lyophilized and dissolved in water (3% of original volume) and insoluble material was removed by centrifugation. Samples were subjected to step-wise precipitation with absolute ethanol (EtOH; 50, 65, and 95% final concentrations), and the precipitates were collected by centrifugation and dissolved in water. Material precipitated by 95% EtOH was analyzed by thin-layer chromatography on silica gel (Baker; 250-μm Si layer) with a 1:1 mixture of 95% EtOH and Milli-Q water as the mobile phase. Selected areas scraped off of developed plates were eluted with 50% EtOH, and siderophore activity in the eluants (diluted at least 1:1 with water) was detected with the CASS reagent. The same fractions also were analyzed on a Perkin-Elmer Sciex API I plus mass spectrometer equipped with an electrospray ionization source (ESI). Five-microliter samples were loop injected into the ESI with a 6:3:0.1 ratio of water, methanol, and formic acid as the carrier at a flow rate of 0.2 ml min^{-1}. The mass spectrometer was scanned from 210 to 700 *m/z* with a 2.0-ms dwell and a 0.2-*m/z* step size.

Recombinant DNA techniques. Plasmid DNA was isolated with a QIAGEN mini kit (QIAGEN, Inc., Valencia, Calif.). Isolation of genomic DNA and natural transformation of *R. solanacearum* to site-specifically replace a wild-type gene with a marked allele were described previously (30). Standard protocols were used for cloning, conjugation, electroporation, competent cell preparation, PCR, and Southern blotting (2).

Creating *R. solanacearum phcA* mutants. To inactivate *phcA* in GMI1000, part of the wild-type gene and the upstream region was PCR amplified from genomic DNA with primers phcA5′ (5′-CTGCCGCCCTTTGTTATCCAC) and phcA3′ (5′-GTACCACCTCACCGCGAAGTC) and cloned into pDrive. A spectinomycin resistance (Sp^r) Ω interposon (45) was then ligated into the unique EcoRV site within *phcA*, resulting in pGMphcAΩ. The *phcA*::Ω construct was subcloned on an EcoRI fragment into pEX18Tc, and the resulting plasmid (pEXphcAΩ) was used in SacB-assisted allelic replacement (22) to introduce the *phcA*::Ω site specifically into the GMI1000 genome. Inactivation of *phcA* was confirmed by PCR amplification and by complementation of GMI1000-PCΩ with wild-type *phcA*. Site-specific inactivation of *phcA* in other *R. solanacearum* strains was achieved by transformation with genomic DNA from AW1-PCΩ or GMI1000-PCΩ or by using pEXphcAΩ as just described.

Creating and characterizing *R. solanacearum* mutants with reduced siderophore activity. Transposon mutagenesis of *R. solanacearum* was performed as described previously (13) with pSup102-Gm::Tn*5* (54). Transformants were selected on BG-nalidixic acid-kanamycin agar plates and then patched onto *Rs*CAS plates. DNA flanking the Tn*5*-B20 insertion in siderophore mutants was recovered from the genome by cloning EcoRI fragments in pBluescript II KS+ and selecting transformants of *E. coli* strain DH5α on LB-ampicillin-kanamycin agar. The Tn*5*ISR primer (5′-GCCGCACGATGAAGAGCAG) was used to sequence *R. solanacearum* DNA flanking the IS*50*R element. Cosmids that complemented selected mutants were recovered by mobilizing an existing library of *R. solanacearum* AW1 genomic DNA en mass from *E. coli* by triparental mating and screening Tc^r transconjugants for increased siderophore production on *Rs*CAS plates.

Nucleotide sequence accession number. The DNA sequence for *ssd* has been submitted to GenBank under accession no. AY541498.

RESULTS

***R. solanacearum* iron-scavenging activity on *Rs*CAS plates.** Screening a collection of preexisting mutants of AW1 revealed that AW1-PC, a *phcA* mutant overproduced iron-scavenging activity compared to the wild type (Fig. 1A). Liquid CAS assays showed that when AW1-PC was cultured in MM liquid medium, the supernatant contained siderophore activity that was equivalent to about 30 μM desferrioxamine mesylate. Each of the nine additional *phcA* mutants that we created also produced more siderophore activity than its parent (Fig. 1B) (data not shown). Therefore, PhcA, the global virulence regulator, appeared to repress production or uptake of siderophore(s). PhcA positively regulates the acyl homoserine lactone quorum-sensing system in strain AW1 (17), so it was possible that *R. solanacearum* could repress siderophore production via its quorum-sensing system as was reported previously for *Burkholderia cepacia* (29). This is not the case, however, because the iron-scavenging activities of AW1-AI8 (*solI*) and AW1-AI3

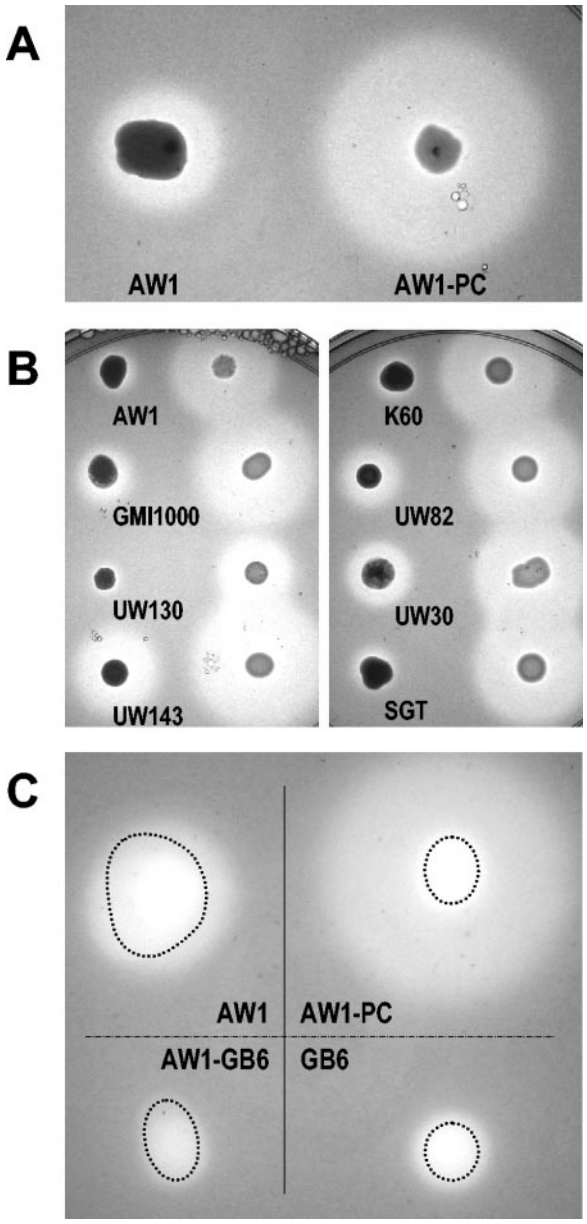


FIG. 1. Iron scavenging by wild-type and mutant *R. solanacearum* strains on CAS indicator plates. The light halos surrounding the bacterial patches are where siderophore has removed iron from CAS, changing its color from blue to orange. (A) AW1-PC (*phcA1*) overproduced siderophore compared to AW1, its wild-type parent (photographed 48 h after stab inoculation). (B) The *phcA* mutants of eight *R. solanacearum* strains overproduced siderophores (photographed 48 h after droplets with 10^4 CFU were deposited on the agar; wild types are to the left and *phcA* mutants are to the right of each pair of columns). (C) Inactivation of *ssd* in AW1-GB6 and GB6 greatly reduced the iron-scavenging ability of these strains compared to their parents. The plate was incubated for 4 days after droplets with 10^4 CFU were deposited on the agar and was then photographed before and after washing off the bacteria. This image shows the plate after bacteria were removed, but with the colony margins indicated by the dotted lines. Pictures were taken with a Canon PowerShot A40 digital camera. Image contrast and brightness were adjusted with Adobe Photoshop, and the figure was assembled with Microsoft PowerPoint.

(*solR*), which cannot synthesize or sense *R. solanacearum* acyl homoserine lactones, respectively, were comparable to that of AW1 (data not shown).

Creation and characterization of mutants with reduced siderophore activity. To screen for transposon mutants that are defective in siderophore production, we used AW1-PC rather than AW1, because it would be easier to detect a decrease in halo size on *Rs*CAS plates. Fifty-one mutants (1.4% of 3,700 mutants) were either siderophore hypoactive or siderophore negative. The siderophore-negative mutants did not grow well when restreaked on *Rs*CAS plates, and further characterization of one such mutant, GB40, revealed that it was a cysteine auxotroph due to inactivation of a *cysI* sulfite reductase ortholog (open reading frame [ORF] *RS02684* in the genome sequence of GMI1000) and the polar effects the transposon had on essential cysteine biosynthetic genes downstream. Consequently, the other mutants that multiplied poorly on *Rs*CAS plates were not studied further.

Strain GB6 was chosen as a representative hypoactive mutant, because it grew well on *Rs*CAS plates but produced only a small orange halo (Fig. 1C). Multiplication of GB6 was comparable to that of AW1-PC in both BG and iron-replete MM media. When cultured in MM liquid medium, GB6 made at least 10-fold-less siderophore activity than did its AW1-PC parent (replicates varied by <20% in five independent experiments). Genomic transformation showed that the Tn*5*-B20 insertion in GB6 was responsible for the siderophore hypoactivity of this strain, and the same procedure was used to site-specifically transfer the mutant allele from GB6 to wild-type AW1, creating strain AW1-GB6. The iron-scavenging activity of AW1-GB6 was almost undetectable on *Rs*CAS plates (Fig. 1C), but there was still a weak color change directly beneath the patch of bacteria.

The *R. solanacearum* DNA flanking the Tn*5*-B20 insertion in GB6 was cloned in pKeGB6 and partially sequenced from a primer site in the transposon. A BLASTn query of the GMI1000 genome showed that the transposon had inserted near the middle of a putative meso-diaminopimelate (DAP) decarboxylase gene. The promoterless *lacZ* gene in the transposon was aligned with the direction of transcription of the putative ORF in GB6. The 3′ end of the ORF was sequenced with a custom primer and pKeGB6 as the template. The 5′ end of the ORF was sequenced with custom primers and a cosmid (pCGB6) that complemented GB6 as the template. Analysis of the assembled sequence (GenBank accession no. AY541498) revealed a 1,242-nucleotide ORF encoding 413 amino acids that are 92% identical and 94% similar to the 413 amino acids encoded by *RS00881* in GMI1000.

A search for conserved domains in the RS00881 protein using reverse position-specific BLAST showed that this putative protein is closely related to LysA DAP-decarboxylase (COG0019.1; E value = 4×10^{-60}), which catalyzes the last step in lysine biosynthesis. Many bacteria have a putative DAP-decarboxylase similar to RS00881. The most similar enzyme with a demonstrated function is LysA from *Pseudomonas aeruginosa* PAO1 (GenBank accession no. P19572), which is only 32% identical and 47% similar over 372 amino acids. Although overall similarity is relatively low among group IV amino acid decarboxylases (e.g., ornithine, DAP, and arginine decarboxylases), there are two consensus motifs (Prosite PDOC00685) (49), both of which are present in RS00881. Motif A, represented by YAxKANxxxxVLxxL (residues 46 to 60), includes the highly conserved lysine thought to be the site of attachment of the pyridoxal-phosphate group. Motif B, represented by GxxLxxLNVGGGIC (residues 223 to 236), contains three consecutive glycine residues proposed to be a part of a substrate binding region. However, because GB6 and AW1-GB6 are not lysine auxotrophs (i.e., they grew on minimal media) and GMI1000 has another ORF (*RS01358*) that appears to encode a DAP-decarboxylase, we propose that RS00881 acts on a substrate specific for siderophore synthesis. We noted also that RS00881 is more like the predicted product of *pvsE* in *Vibrio parahaemolyticus* (44% identical, 60% similar), which is essential for biosynthesis of the polycarboxylate siderophore vibrioferrin (56), and to a putative decarboxylase (42% identical, 57% similar) encoded within a gene cluster required for biosynthesis of achromobactin, a polycarboxylate siderophore made by *Erwinia chrysanthemi* (GenBank AAL14568; unpublished). Therefore, *RS00881* was designated as the siderophore synthesis decarboxylase (*ssd*) gene.

TABLE 2. Effect of iron on siderophore production, expression of the *ssd*::*lacZ* transcriptional fusion, and effect of sodium citrate on multiplication of *R. solanacearum* in minimal medium

Strain	Mutation	[Fe] (μM)[a]	CAS activity[b]	β-Galactosidase activity (Miller units)[c]	Turbidity (OD_{600}) ± citrate[d]			
					24 h		48 h	
					−	+	−	+
AW1	None	0	+	ND	0.04	0.36	0.79	1.92
		10	−	ND	0.13	0.47	3.46	3.12
AW1-GB6	*ssd*	0	−	85 ± 37	0.01	0.20	0.01	0.76
		10	−	2.4 ± 1.5	0.02	0.30	0.15	3.40
AW1-PC	*phcA1*	0	++	ND	0.15	0.68	2.13	2.13
		10	−	ND	0.41	1.70	2.62	3.02
GB6	*phcA1 ssd*	0	−	483 ± 37	0.01	0.29	0.01	1.34
		10	−	2.6 ± 1.2	0.02	0.73	0.45	2.73

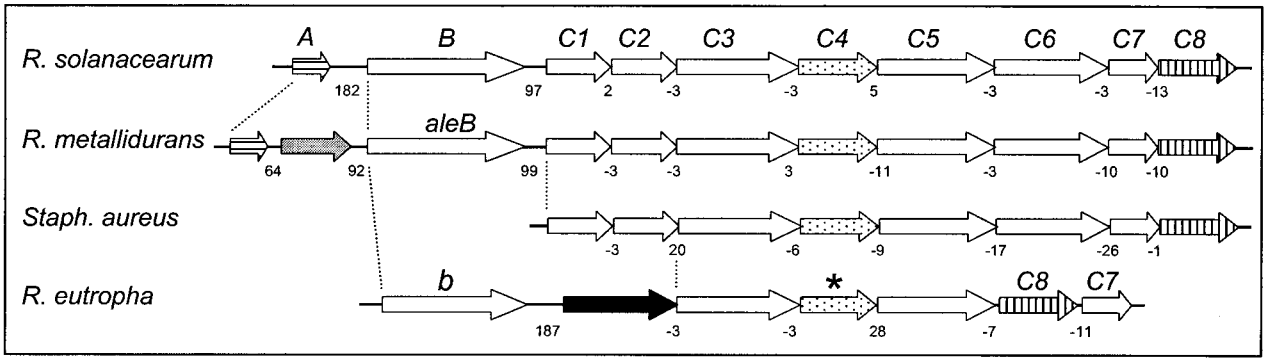
[a] Final concentration of $FeCl_3$ added. The concentration of Fe^{3+} inherent in the minimal media was not determined.

[b] CAS assay for siderophore-mediated iron chelation. Symbols indicate A_{630} ranges: −, > 0.9; +, from 0.18 to 0.3; ++, from 0.08 to 0.18. Similar values were observed in MM and MMΔ.

[c] β-Galactosidase activity encoded by an *ssd*::*lacZ* fusion determined for cells cultured in MM. Results are the mean ± standard deviation of three independent experiments with two replicates of each culture. ND, not done.

[d] Turbidity was determined at 48 h. Cultures with an OD_{600} of >1 were diluted 10-fold prior to measurement. −, without citrate; +, with citrate at 0.5 mM. Results are from one experiment that was representative of three independent experiments; values are the mean of duplicate tubes.

The effect of iron on siderophore production by *R. solanacearum*, expression of the *ssd*::*lacZ* fusion in AW1-GB6 and GB6, and bacterial multiplication was studied in liquid minimal medium (Table 2). As expected, AW1 produced less siderophore activity than did AW1-PC when no iron was added, and the addition of ≥3 μM Fe^{3+} repressed siderophore production by both strains. The *ssd* mutants AW1-GB6 and GB6 produced no detectable siderophore activity when cultured in MM or MMΔ (i.e., with or without sodium citrate, respectively). In the presence of 10 μM Fe^{3+}, the *ssd*::*lacZ* fusion was transcribed weakly, but transcription increased about 30-fold in AW1-GB6 (*ssd*) and about 200-fold in GB6



	R. solanacearum GMI1000			*R. metallidurans* CH34				*S. aureus* MW2				*R. eutropha* H16			
Code	ORF	Putative Function	COG	ORF	Score	% ID	% Tot	ORF	Score	% ID	% Tot	ORF	Score	% ID	% Tot
A	872	ECF sigma factor	1595	4332	160	32	73	no ortholog in cluster				no ortholog in cluster			
B	873	OM siderophore receptor	1629	AleB	2650	64	97	no ortholog in cluster				126	175	22	66
C1	874	Cysteine synthetase	0031	4335	1139	69	94	0089	949	56	93	no ortholog in cluster			
C2	875	Ornithine cyclodeaminase	2323	4336	1340	77	97	0090	1033	58	97	no ortholog in cluster			
C3	876	Siderophore synthetase subunit	4264	4337	1349	48	94	0091	531	30	93	124	189	23	80
C4	877	MFS transporter	0477	4338	624	39	94	0092	399	26	93	123	* transporter		
C5	878	Siderophore synthetase subunit	4264	4339	1483	51	97	0093	602	32	81	122	386	27	68
C6	879	Siderophore synthetase subunit	4264	4340	1834	60	98	0094	899	36	95	no ortholog in cluster			
C7	880	Hydroxyacid aldolase	3836	4341	669	58	93	0095	495	42	91	120	431	42	91
C8	881	Ssd (sid. synth. decarboxylase)	0019	4342	1287	63	95	0096	946	47	93	121	848	46	93

FIG. 2. Putative locus for scavenging of iron(III) in *R. solanacearum* GMI1000 and related loci in *R. metallidurans* and *S. aureus*, two bacteria that produce the polycarboxylate staphyloferrin B. A related locus in the *R. eutropha* H16 megaplasmid (AY305387) that might encode iron(III) acquisition is also shown. The upper panel shows the size and arrangement of the ORFs. Open and patterned arrows denote genes potentially required for regulation, biosynthesis, and export of staphyloferrin B or uptake of ferri-staphyloferrin B. Filled arrows denote genes not involved in these processes. The *R. eutropha* putative siderophore receptor (gene b) has low homology to *R. solanacearum* gene B, and this is indicated by its having a lowercase letter. An asterisk designates that the *R. eutropha* putative MFS transporter gene is not homologous to *R. solanacearum* gene C4. The number of bases between the ORFs is indicated below each line (negative numbers indicate overlap of the ORFs). The lower panel gives selected details for the ORFs in GMI1000 and how similar the encoded proteins are to orthologs in three other bacteria. COG, cluster of orthologous group number; Score, BLASTp score; % ID, percentage of identical amino acids; % Tot, percentage of total amino acids aligned; sid. synth., siderophore synthesis.

(*ssd phcA1*) when no iron was added. Therefore, in addition to being repressed by iron, transcription of *ssd* also is repressed by the PhcA global virulence regulator. The effect of iron on multiplication of *R. solanacearum* was strongly influenced by the presence of sodium citrate (Table 2). When compared to their parental strains, multiplication of the *ssd* mutants in medium without citrate was strongly inhibited whether or not 10 μM Fe^{3+} was added. All four strains multiplied better when sodium citrate was added, especially when 10 μM Fe^{3+} also was present. Due to the low concentration of sodium citrate added (500 μM), it is probably acting as an exogenous iron chelator rather than as a nutrient for *R. solanacearum*. All of these results are consistent with *ssd* encoding a protein that is essential for PhcA-regulated siderophore production by *R. solanacearum*.

Four independent assays showed that the ability of AW1-GB6 to wilt and kill tomato plants was comparable to that of its wild-type parent (data not shown). The virulence of GB6 was not tested, because its parental strain is avirulent (4). When AW1 was cultured in xylem sap recovered from decapitated tomato plants, it multiplied rapidly and did not produce siderophore activity detectable by the liquid CAS assay.

Evidence that *R. solanacearum* does not make schizokinen. Siderophore biosynthesis generally is encoded by an operon, so we expected to find genes for production of the dihydroxamate schizokinen adjacent to *ssd*. In the annotated sequence of GMI1000 (48), *ssd* is the terminal gene in a cluster of seven additional ORFs beginning with a *cysK* ortholog (Fig. 2). These eight genes probably comprise an operon, because the ORFs overlap or are separated by ≤5 nucleotides. Although ORF analysis indicated the presence of three genes for putative siderophore synthetase subunits (orthologs of IucA and IucC) like those required for synthesis of the dihydroxamate aerobactin by *E. coli* (12), the operon lacks genes for a monooxygenase and an acetylase (orthologs of IucD and IucB, respectively), which commonly synthesize the hydroxamate moiety (15, 34). Therefore, this operon appeared to lack some of the genes expected for schizokinen biosynthesis.

Comparative genetic analyses (see Discussion) suggested that the *cysK-ssd* operon in GMI1000 might instead be for biosynthesis of staphyloferrin B, a polycarboxylate siderophore. We first tested this hypothesis by comparing the siderophores made by *R. solanacearum* AW1-PC, *R. metallidurans* CH34 (staphyloferrin B) (38), and *B. megaterium* ATCC 19213 (schizokinen), using standard chemical tests for the presence of hydroxamate or catecholate moieties (Table 3). The AW1-PC siderophore and staphyloferrin B were negative in both tests, whereas schizokinen was positive for hydroxamates as expected. Wild-type AW1 and all seven of the other wild-type *R. solanacearum* strains shown in Fig. 1B also produced a siderophore that was negative in both tests (data not shown).

We next used a siderophore bioassay (44) to determine

TABLE 3. Chemical characteristics of selected siderophores and their effect on multiplication of AW1-GB6 (*ssd*) in iron-deficient minimal medium

Siderophore source[a]	Functional group[b]		Turbidity (OD_{600})[c]	
	Hydroxamate	Catechol	Fe−	Fe+
None	ND	ND	0.35	ND
R. solanacearum AW1-GB6	ND	ND	0.39	ND
R. solanacearum AW1-PC	−	−	3.30	ND
R. metallidurans CH34	−	−	3.37	ND
B. megaterium ATCC 19213	+	−	0.009	3.60
Desferrioxamine (10 μM)	+	−	0.007	2.01

[a] Bacteria were removed from stationary-phase cultures (e.g., 48 h old) by centrifugation and the supernatant was filter sterilized.

[b] Hydroxamates and catechols were detected with the Csàky and Amow tests, respectively. ND, not done.

[c] Bioassay tubes contained 0.5 ml of MMΔ (amended with 0.25 % [wt/vol] non-deferrated yeast extract, 150 μM 2,2′-dipyridyl, and 20 μg of kanamycin ml^{-1}) and 10^6 CFU of AW1-GB6 ml^{-1}. Appropriate tubes received 10 μl of a culture supernatant or 10 μl of 0.5 mM desferrioxamine mesylate. Selected tubes also contained 20 μM $FeCl_3$. Turbidity of AW1-GB6 was determined at 48 h; cultures with an OD_{600} of >1 were diluted 10-fold prior to measurement. Results are from one experiment that was representative of three independent experiments; values are the mean of triplicate tubes.

which iron chelators supported the multiplication of *R. solanacearum* AW1-GB6 in a modified MMΔ that contained iron but was made iron limiting by addition of the Fe^{2+} chelator 2, 2′-dipyridyl (Table 3). In the absence of exogenous siderophore, AW1-GB6 multiplied to a maximum turbidity of about 0.4 at an optical density of 600 nm (OD_{600}), and as expected addition of sterile supernatant (a 50-fold dilution) from an AW1-GB6 culture had no effect. However, the siderophores present in the supernatants of both AW1-PC and *R. metallidurans* CH34 increased the maximum turbidity 10-fold. All seven of the other *R. solanacearum* wild-type strains shown in Fig. 1B produced a siderophore that similarly promoted multiplication of AW1-GB6 (data not shown). In contrast, both the schizokinen present in the supernatant of *B. megaterium* ATCC 19213 and desferrioxamine mesylate suppressed multiplication of AW1-GB6, unless excess Fe^{3+} was added (Table 3). These results showed that *R. solanacearum* AW1-GB6 does not utilize schizokinen, as one would expect from the results of Budzikiewicz et al. (5), but can utilize staphyloferrin B produced by *R. metallidurans* and the siderophore made by multiple *R. solanacearum* strains (including the type strain ATCC 11696 [=K60]).

Bacteria often can utilize exogenous siderophores that they cannot synthesize, so the above results are insufficient to conclude that *R. solanacearum* produces staphyloferrin B. To physically characterize the *R. solanacearum* siderophore, we first determined that both it and staphyloferrin B were soluble in 65% EtOH, but were precipitated by 95% EtOH (the "active fraction"). The material precipitated by 95% EtOH from a supernatant of GB6 (*ssd*) had no siderophore activity. Second, thin-layer chromatography analysis of the CH34 and AW1-PC active fractions showed that the siderophores comigrated as a single spot ($R_f \simeq 0.75$). Third, the mass spectrum of the CH34 active fraction had a large peak at 449 *m/z*, which is the correct protonated molecular ion for staphyloferrin B (14). Peaks at 431, 387, and 369 *m/z* may correspond to those present in the spectrum for pure staphyloferrin B, whereas peaks at 471 and 487 *m/z* are probably the sodium and potassium salts of the siderophore, respectively. The spectrum from the AW1-PC active fraction also had peaks at 449 and 487 *m/z*, suggesting the presence of staphyloferrin B, whereas these are the only peaks missing in the spectrum of the comparable, siderophore inactive fraction from GB6 (Fig. 3). Taken together, our biological and physical tests indicate that AW1-PC and the other *R. solanacearum* strains tested produce staphyloferrin B.

DISCUSSION

PhcA is an environmentally responsive transcriptional regulator that controls expression of an unknown number of genes in *R. solanacearum*, many of which promote virulence (50). The activity of PhcA is controlled by a novel confinement-sensing system that uses 3-hydroxypalmitic acid methyl ester as the autoinducer (17). Genes positively controlled by PhcA are expressed best at high cell density and include those for EPS1 biosynthesis, several extracellular plant cell wall-degrading enzymes, and a typical acyl homoserine lactone quorum-sensing system. Genes negatively regulated by PhcA have been more difficult to identify because they are expressed well only at low cell density, but include those for one extracellular plant cell wall-degrading enzyme, flagellar motility, and type IV pilus-dependent twitching motility (30, 50). In this study, we found that staphyloferrin B production is another trait negatively regulated by PhcA, because *R. solanacearum phcA* mutants uniformly overproduced siderophore activity relative to their wild-type parents. As expected, siderophore production also was eliminated by the presence of >3 μM iron in the growth medium, presumably via repression by Fur. That transcription of *ssd* is repressed by both PhcA and iron(III) fits the observed phenotype and supports our conclusion that *ssd* is involved in staphyloferrin B biosynthesis. However, we could not predict the substrate of Ssd or its position in a biosynthetic pathway.

The putative *cysK-ssd* operon does not appear to code for enzymes necessary to synthesize a dihydroxamate siderophore like schizokinen, which *R. solanacearum* ATCC 11696 reportedly produces (5). A search for homologs of the *cysK-ssd* operon in various databases revealed a very similar region in the genome of *R. metallidurans* CH34 that is involved in siderophore production (Fig. 2) (20). Originally called alcaligen E, the siderophore made by CH34 was later identified as the polycarboxylate staphyloferrin B (38). Transposon insertions in the *R. metallidurans* CH34 *cysK-ssd* region eliminated siderophore production and the *aleB* gene immediately upstream of this operon encodes the outer membrane siderophore receptor for staphyloferrin B (20). The ORF (*RS00873*; gene B in Fig. 2) at this location in GMI1000 is 64% identical to AleB. Therefore, we postulated that *R. solanacearum* uses some or all of the *cysK-ssd* operon to synthesize staphyloferrin B instead of, or in addition to, schizokinen.

Characterization of the siderophores made by *R. solanacearum* showed that eight wild-type strains (including strain K60, which we provided to the American Type Culture Collection in 1999 to replace their deteriorated culture of type strain 11696) did not make detectable amounts of schizokinen under our conditions. In addition, schizokinen repressed multiplication of AW1-GB6 (*ssd*) in an iron-restricted medium, apparently by further restricting iron availability. In contrast, staphyloferrin

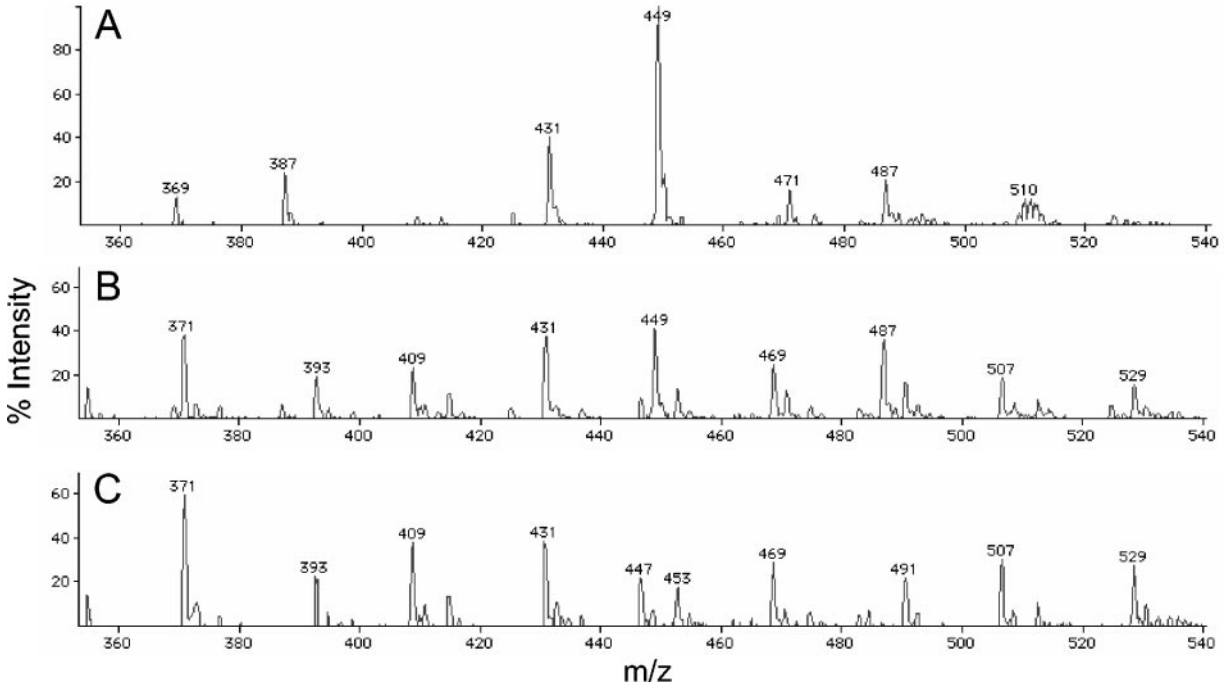


FIG. 3. Mass spectra of culture supernatants to detect staphyloferrin B. Samples were partially purified and concentrated about 200-fold prior to mass spectroscopy. (A) *R. metallidurans* CH34, which produces staphyloferrin B, has a peak at 449 *m*/*z* that is consistent with the molecular ion $[M + H]^+$ of this siderophore. (B) *R. solanacearum* AW1-PC (*phcA1*), which is siderophore positive, also has a peak at 449 *m*/*z*. (C) *R. solanacearum* GB6 (*phcA1 ssd*), which is siderophore negative, lacks the peak at 449 *m*/*z*. See the text for further analysis of the spectra. The spectra for both AW1-PC and GB6 were recorded about 6.7 min after sample injection. The spectra shown had the background electronically subtracted, and the signal from the GB6 sample was amplified slightly compared to the other two samples.

B supported multiplication of AW1-GB6 in iron-restricted medium and the siderophore made by *R. solanacearum* AW1-PC appeared to have the same the mass (448 Da) as staphyloferrin B. One explanation for the discrepancy between our results and those of Budzikiewicz et al. (5) is that they characterized the siderophore made by a bacterium that was not *R. solanacearum* ATCC 11696.

All seven other proteins encoded by the *R. solanacearum cysK-ssd* operon are orthologous to proteins with known functions, but only four have predictable roles in siderophore production. Three proteins (Fig. 2, proteins C3, C5, and C6) are similar to siderophore synthetase subunits IucA and IucC (12) and probably create amide linkages between the different constituents. The fourth protein with a predictable role is C4 (Fig. 2, RS00877), because it is related to the *Bacillus subtilis* multidrug exporter Bmr (GenBank accession no. P33449) and multiple tetracycline exporters in the major facilitator superfamily (MFS) (41, 43). MFS transporters are integral membrane proteins that transport structurally diverse small molecules using the proton motive force. Although the similarity of protein C4 to other MFS transporters is relatively low (<25% identity), it is predicted to have the 12 transmembrane segments and all five conserved amino acid motifs (A, B, C, D2, and G) typical of the DHA12 family of drug:H^+ antiporters (43). Little is known about export of siderophores, but export of the catecholate enterobactin from *E. coli* requires EntS, a DHA12 family protein (18), and a putative MFS transporter may be necessary for efficient export of the polycarboxylate siderophore vibrioferrin by *V. parahaemolyticus* (56).

Production of polycarboxylate siderophores may be common in *Ralstonia* species. For example, *Ralstonia pickettii* DSM 6297 reportedly produces the polycarboxylate *S*, *S*-rhizoferrin (39). In addition, *Ralstonia eutropha* H16 has a gene cluster (ORFs PHG120 to PHG126) on a self-transmissible megaplasmid that might be involved in siderophore biosynthesis (51). This gene cluster is smaller than that in *R. solanacearum* and only contains orthologs for *aleB* and genes C3, C5, C7, and C8 (Fig. 2). The *R. eutropha* gene cluster also appears to encode a MFS transporter in the DHA12 family, but its amino acid sequence is unrelated to *R. solanacearum* protein C4. Because the gene cluster in H16, including the putative MFS transporter, is similar to a region in *V. parahaemolyticus* that is required for biosynthesis and export of vibrioferrin (56), it is quite possible that *R. eutropha* makes a similar polycarboxylate siderophore.

Surprisingly, our search for homologs of the *cysK-ssd* operon also revealed a comparable region involved in production of a siderophore in the genome of *Staphylococcus aureus* MW2 and four other *S. aureus* strains (10). The *sbn* operon in *S. aureus* is colinear with that in *R. solanacearum* and *R. metallidurans* (Fig. 2), but contains a ninth ORF of unknown function downstream of the *ssd* ortholog. The high degree of amino acid similarity of orthologous *R. solanacearum* and *S. aureus* proteins is all the more remarkable, given that the genomic G+C contents of these organisms are about 67 and 32%, respectively. Although most *S. aureus* strains make the polycarboxylate siderophores staphyloferrin A and staphyloferrin B (21), Dale et al. (10) reported that the *sbn* operon in a *fur* mutant of strain RN6390 was required for production of an uncharacterized siderophore

tentatively called staphylobactin that has a mass >300 Da larger than either staphyloferrin A or B. These results are unexpected given the discussion above concerning staphyloferrin B production by *R. solanacearum* and *R. metallidurans*. Structural characterization of staphylobactin and additional genetic studies with *S. aureus* strains known to produce staphyloferrin B are needed to resolve this issue.

The requirement for iron during bacterial pathogenesis has been studied primarily in animal pathogens, so much less is known about this relationship in plant pathogens (16, 47). *Erwinia amylovora* and *E. chrysanthemi* are the only two phytopathogenic bacteria known to require siderophores to be fully pathogenic. *E. amylovora* CFPB1430 produces desferrioxamines E and D_2 (16), and mutants that did not synthesize these siderophores exhibited reduced virulence on apple blossoms (11). *E. chrysanthemi* 3937 produces achromobactin and chrysobactin (16, 37), and mutants unable to make either siderophore cause lesions at an inoculation site but not systemic soft rot on African violet (16). In contrast, *Erwinia carotovora* WC3105 requires neither aerobactin nor chrysobactin to macerate potato tuber tissue or cause aerial stem rot of potato (3) and two *Pseudomonas syringae* strains do not require a pyoverdin-like siderophore to infect cherry fruit or to colonize bean leaf surfaces (28, 31).

Virulence assays showed that staphyloferrin B production is not required by *R. solanacearum* AW1 for pathogenesis when bacteria were applied to unwounded roots of young tomato plants. That siderophore production is nonessential in planta can be explained by our finding that tomato xylem sap is not iron limiting. Indeed, tomato xylem sap has been reported to contain >5 μM iron (58), which would suppress siderophore production if the iron is present in a form that *R. solanacearum* can utilize (e.g., ferric citrate). It is noteworthy that tomato and tobacco plants engineered to overproduce human lactoferrin, an iron-binding glycoprotein, display enhanced resistance to *R. solanacearum* strain K60 (27, 59). It would be interesting to test whether *R. solanacearum* produces staphyloferrin B in these transgenic plants, because this result would support iron withholding as the mechanism of resistance.

One cannot conclude, however, that siderophore production is never important for *R. solanacearum*, because our assay does not require bacteria to survive in soil for more than 1 week outside of its host. In natural settings, lower numbers of the pathogen would have to compete with other soil microbes for iron, especially in the nutrient-rich zone surrounding plant roots (the rhizosphere) (33, 53, 57). The importance of siderophore production for survival and function in soil environments has been examined for some bacteria that have biocontrol potential or that stimulate plant growth. In some studies with fluorescent pseudomonads, effective competition for iron in the rhizosphere contributes to survival, root colonization, and disease suppression (23, 31, 46), but other studies report the opposite conclusion (26, 42). Studies employing bacteria engineered to be biosensors of iron availability have repeatedly shown that this element is unequally distributed in soil and on plant surfaces and that a minority of the test population inhabits low-iron microsites (25, 32, 35). Since so many variables could affect whether *R. solanacearum* needs siderophores for survival or pathogenesis in natural settings, this aspect of pathogen biology will be difficult to evaluate definitively.

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